International Journal of Agricultural Technology 2017 Vol. 13(7.1):1231-1244
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Isolation and Screening of Endophytic Bacteria against Rice Blast Pathogen

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Taksaphorn Changmuang, Potjana Sikhao and Prommart Koohakan (2017). Isolation and Screening of Endophytic Bacteria against Rice Blast Pathogen. International Journal of Agricultural Technology 13(7.1): 1231-1244.

This study aimed for isolation endophytic bacteria from leaves, stems and roots of healthy rice and screening their potential to promote plant growth and inhibit *Pyricularia oryzae* caused rice blast disease. Forty-six isolates were obtained from this experiment. Initially, the effect on rice seedling were tested. It was found that 41 isolates showed their ability to promote plant growth that was referred to benefit isolates. Isolate Bar917 had the highest seedling vigor index that was 128.42 %, following by isolate sus617 at 128.03%. Then the benefit isolates were also tested their efficiency to inhibit *P. oryzae* by dual culture technique. It was found that all benefit isolates could inhibit *P. oryzae*. The group of isolates that was 60% inhibition higher than control including sus217, sur317, Bas417 and Bar917, which was 66.80, 66.66, 64.86 and 61.11% respectively. Characterization of benefit isolates by gram staining and 3% KOH test, found that most of them were gram-positive bacteria. From this experiment, we selected some benefit isolate for further study on seed bio-priming to improve its efficiency for rice production in the future.

Keywords: endophytic bacteria, rice blast, *Pyricularia oryzae*

Introduction

Rice is one of the most important economic crops particularly in Asia where almost half of the population relies on it as the main food (Hegde and Hedge, 2013), but rice production encountered a major problem in the field of plant disease that is rice blast disease. Rice blast disease is caused by the Ascomycetous fungus *Pyricularia oryzae* (Couch and Kohn, 2002) Rice blast symptoms can occur on all aboveground parts of the plant and is observed at earlier growing stages until the final grain production, percentage of seeds decreased causing economic damage. (Yorionori and Thurston 1974) This disease has been controlled with fungicides, though this method is the most effective control but the use of chemical frequently may affect on environment

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and chemical residues that harmful to both farmers and consumers. Leading to some researcher have focused their efforts on developing alternative inputs to synthetic chemicals for controlling diseases, and biological control is an interesting alternative

Currently, biological control methods used for disease control has been more interesting. For example using of antagonistic microorganisms such as *Trichoderma hazianum* or *Bacillus subtilis*. There is also the attractions for endophytic bacteria, which are bacteria live inside the plant tissue but it does not cause plant disease. (Hallman *et al*., 1997) de Matos Nogueira *et al*., (2001) reported that endophytic bacteria could have the capacity to control plant pathogen and plant growth promotion. The purpose of this study was isolation and screening of endophytic bacteria against rice blast pathogen and growth promotion to rice and selected some benefit isolate for further study on seed bio-priming to improve its efficiency for rice production in the future.

Objectives: Isolation and screening of endophytic bacteria against rice blast pathogen and growth promotion for further study on seed bio-priming.

Materials and methods

Isolation of Pyricularia oryzae and pathogenicity tests

Cultures of *P. oryzae* used in this study were isolated from infected rice by single spore isolation and recieved from Plant Pathology Laboratory, faculty of Agricultural KMITL. The isolates were transferred to petri dishes containing rice flour agar (RFA) and incubated at 37 ˚C for 14 days. After 14 days, sporulations were induced by adding 2 ml of sterile distilled water into the petri dishes of *P. oryzae* then using L-Shape glass rod to scrap on the surface of culture media, and prepared to spore suspension at concentration of 10^5 spore / ml. Then 80 ml of the suspension was mixed with 20 ml of 2 % gelatin solution and sprayed on seedling rice. Disease severity was evaluated by scoring based on 0-9 ordinal scale (IRRI 1996) where: 0= No of lesions, 1= Small brown speaks of pin point size or large brown speak, 2= Small round dish to slightly elongated necrotic grey spots about 1-2 (mm) in diameter with distinct brown margin lesions are mostly found on lower leaves, 3= Lesion type is same in scale 2 but significant number of lesion are one on upper leaves, 4= Typical susceptible blast lesion, 3 mm or longer infecting lesions than 2% of leaf area, 5= Typical blast lesion infecting 2-10 % of the leaf area, 6= Typical blast lesion infecting 11-25 % of the leaf area, 7= Typical blast lesion infecting 26-50% of the leaf area, 8= Typical blast lesion infecting 51-75% of the leaf area many leaves are dead, and 9= More than 75% leaf are affected. The most violent

isolate was chose for next experiments. The experimental design was completely randomized design (CRD) with 4 replications.

Screening and selection the potential isolate

Promotion activities on rice seedling

The former isolate of endophytic bacteria that was reported on the ability to control rice disease (Koohakan and Konrangdee, 2015) and new one isolated from healthy rice were tested. All isolates were subcultured on nutrient agar (NA) and incubated for 48 hours. Then they were transferred to nutrient broth (NB) and incubated on ratary shaker for 48 hours. The culture was centrifuged at 5000 rpm for 10 minute, cell culture pellet was diluted with normal saline and adjusted the concentration by compared the turbidity with McFarland standard No. 0.5. Then the suspension was inoculated to rice seedling followed by Koohakarn and Konrangdee (2015). After 7 days of inoculation with endophytic bacteria, the growth parameter of seedlings such as seed germination, seedling height, stem weight, root weight, number of leaves and total weight were collected. Data were calculated for growth index of seedling vigor index (svi) as following formular

$$\text{svi} = \text{Average germination percentage} \times \text{Average weight per plants}$$

$$\%\ \text{svi} = \frac{\text{svi of teatment}}{\text{svi of control}} \times 100$$

Which isolate has % SVI $\geq$ 100% indicated the ability to promote plant growth and referred to benficial isolates for further tested.

Inhibitory activity against rice blast pathogen

The beneficial isolates of endophytic bacteria were grown on NA medium and incubated at room temperature for 24 hours. The pathogen, *P. oryzae*, was cultured on RFA for 14 days. Inhibition effect was evaluated by using dual culture test on petri dish containing PDA media. The pathogen agar plug was placed on the center of culture medium for 4 days before endophytic bacteria was streaked. Then the tested isolates were streaked 2 cm. length form the edge pararell on the left and right sides of the pathogen and incubated at room temperature. Evaluation of the mycelial growth inhibition was done when the pathogen in control grown full in the petri dish. The mycelial growth inhibition rate (IR) was calculated using the formula as follow: [(C2-C1)/ C2 x 100 where C2: diameter of the pathogen colony on control plate and C1: diameter of the pathogen colony on the inhibition plate. The experimental design was completely randomized design (CRD) with 4 replications.

Data analysis

The results data were subjected to analysis of variance and treatment means were separated by Duncan's multiple range test to assesssignificant ($P \leq 0.05$) differences among means (Duncan, 1955)

Results

Isolation of Pyricularia oryzae and pathogenicity tests

BTN6001 isolated from infected rice was morphological study to confirm as *P. oryzae* and pathogenicity test compared with 3 former isolates RBR55001, UBN195271 and BKK55003 obtained from Plant Pathology Laboratory, KMITL. The results found that RBR55001 had the highest disease severity at level 8, followed by isolate BKK55003 at level 6 isolates, UBN195271 and BTN6001 at levels 5 and 4, respectively. (Figure 1) Therefor, RBR55001was chose for further test.

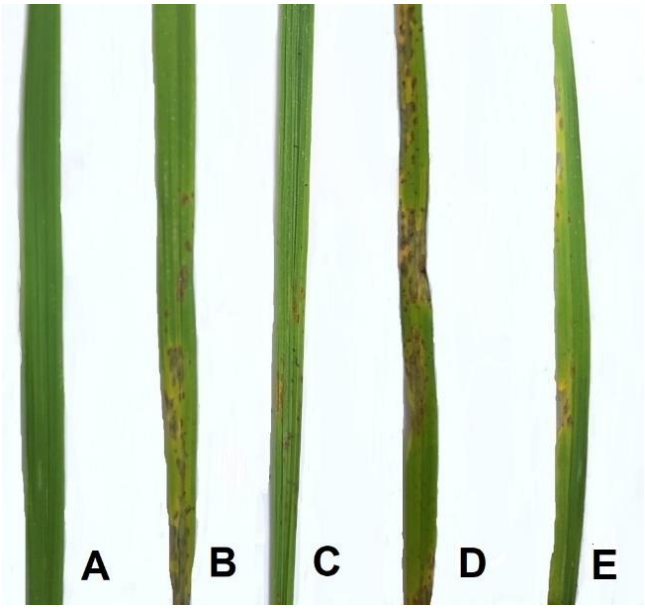

Figure 1. Rice leaves inoculated with isolated of *P. oryzae* (A=control; B=BKK55003; C=UBN195271; D=RBR55001E= BTN6001)

Screening and selection the potential isolate

Promotion activities on rice seedling

Endophytic bacteria tested in this experiment consist of the 15 previous isolates reported by Koohakan and Konrangdee (2015) for their ability to promote rice growth and inhibit rice blast pathopgen (BEdStUTI001, BEdStUTI002, BEdStUTI003, BEdStUTI004, BEdStUTI006, BEdStRBR001, BEdStRBR002, BEdStRBR003, BEdStRBR005, BEdStSPB001, BEdStSPB002, BEdStSPB003, BEdStSPB004, BEdStSPB005, BEdSTPB001) and 31 recent islolates that were isolated from healthy rice (SnR117, SnS217, SnS317, Sn417, SnR517, SnR617, SnS717, SnR817, SnS917, SnS1017, SuR117, SuS217, SuR317, SuS417, SuR517, SuS617, SuR717, Su2R117, Su2S217, Su2R317, Su2L417, SuS2R517, Su2R617, BaR217, BaR517, BaL517, BaS417, BaR617, BaL717, BaR917, BaR1017). Their characteristics were observed under microscope, gram staining and 3%KOH were also tested. The results showed that there were 39 isolates of Gram-positive and 7 isolates of Gram-negative and showed different colony characteristics according to Table 1 and Figure 2

Table 1. Characterization of endophytic bacteria

Isolates	Colony on NA				3%KOH test	Gram staining	Shape style
	Colors	Shape	Margin	Surface			
1.BEdStUTI001	white	circular	entire	mucoid	+	+	107.74
2.BEdStUTI002	yellow	circular	entire	mucoid	+	+	111.61
3.BEdStUTI003	yellow	circular	entire	mucoid	-	-	104.37
4.BEdStUTI004	white	circular	entire	mucoid	-	-	101.76
5.BEdStUTI006	cloudy white	circular	entire	smooth	+	+	114.47
6.BEdStRBR001	light yellow	circular	entire	mucoid	+	+	100.98
7.BEdStRBR002	cloudy white	circular	entire	rough	+	+	111.59
8.BEdStRBR003	egg	circular	entire	mucoid	+	+	110.14
9.BEdStRBR005	white	circular	entire	mucoid	+	+	105.82
10.BEdStSPB001	yellow	circular	entire	mucoid	+	+	105.33
11.BEdStSPB002	cloudy white	circular	entire	smooth	+	+	105.33
12.BEdStSPB003	yellow	circular	entire	rough	+	+	116.40
13. BEdStSPB004	white	circular	entire	mucoid	+	+	121.21
14.BEdStSPB005	light yellow	circular	entire	mucoid	-	-	111.11
15.BEdSTPB001_r	egg	circular	entire	rough	+	+	114.47
16.SnR117	white	circular	entire	mucoid	-	-	96.54

Isolates	Colony on NA				3%KOH test	Gram staining	Shape style
	Colors	Shape	Margin	Surface			
17.SnS217	cloudy white	irregular	undulate	smooth	+	+	106.30
18.SnS317	cloudy white	circular	entire	smooth	+	+	104.37
19.SnS417	egg	circular	entire	mucoid	+	+	104.18
20.SnR517	white	circular	entire	mucoid	+	+	104.37
21.SnR617	white	circular	entire	mucoid	-	-	119.76
22.SnS717	white	circular	entire	mucoid	+	+	102.93
23.SnR817	white	circular	entire	mucoid	+	+	102.45
24.SnS917	egg	circular	entire	mucoid	+	+	114.89
25.SnS1017	yellow	circular	entire	mucoid	+	+	98.24
26.SuR117	cloudy white	circular	entire	mucoid	+	+	102.23
27.SuS217	cloudy white	circular	entire	mucoid	+	+	97.07
28.SuR317	cloudy white	circular	undulate	smooth	+	+	121.21
29.SuS417	cloudy white	circular	entire	mucoid	+	+	117.89
30.SuR517	white	circular	entire	mucoid	+	+	103.72
31.SuR617	white	circular	entire	mucoid	+	+	128.03
32.SuR717	white	circular	entire	mucoid	+	+	102.93
33.Su2R117	egg	circular	entire	mucoid	+	+	105.51
34.Su2S217	cloudy white	circular	undulate	rough	+	+	109.89
35.Su2R317	white	circular	entire	mucoid	-	-	103.89
36.Su2L417	white	circular	entire	mucoid	+	+	117.84
37.Su2R517	cloudy white	circular	undulate	rough	+	+	109.66
38.Su2R617	white	circular	entire	mucoid	-	-	109.66
39.BaR217	white	circular	entire	mucoid	+	+	128.42
40.BaR317	cloudy white	circular	entire	rough	+	+	105.82
41.BaS417	cloudy white	circular	undulate	rough	+	+	115.92
42.BaL517	egg	circular	entire	smooth	+	+	103.17
43.BaR617	white	circular	entire	mucoid	+	+	106.01
44.BaL717	cloudy white	circular	undulate	rough	+	+	119.28
45.BaR917	cloudy white	circular	entire	smooth	+	+	128.90
46.BaR1017	brown	circular	undulate	mucoid	+	+	116.30

The 46 isolatees of endophytic bacteria were tested their effects on the growth of rice seedling. It was found that the number of leaves and survival of

seedlings in all isolates were not significant difference compared with control, but stem height, shoot weight, root weight and total weight of seedling were significant difference. Acording to %SVI, we found that there were 43 isolates with %SVI higher than control, which referred to beneficial isolates to promote rice growth. Among the beneficial isolates, Bar917 had the highest %SVI that was 128.90 % compared with control (Table2).

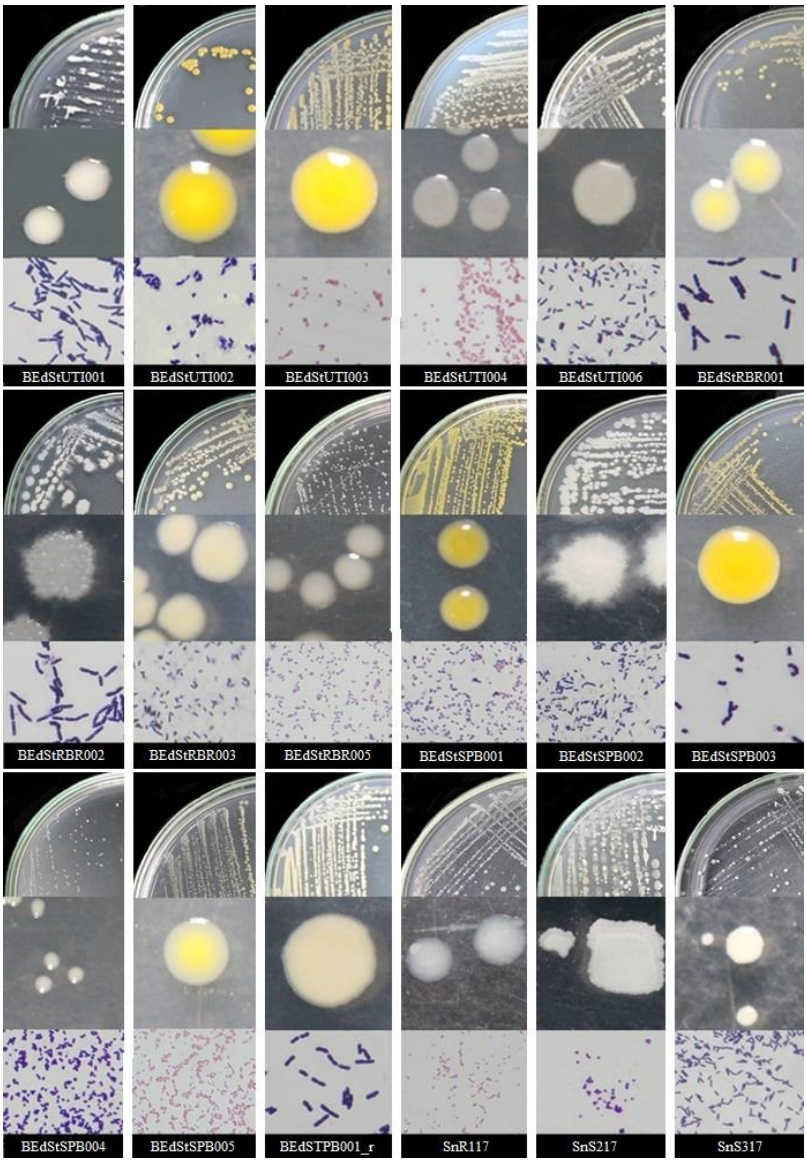

Figure 2. Morphology of endophytic bacteria from healthy rice plant **(**Above = colony on NA, Middle = colony at 6.7X and Under = gram staining)

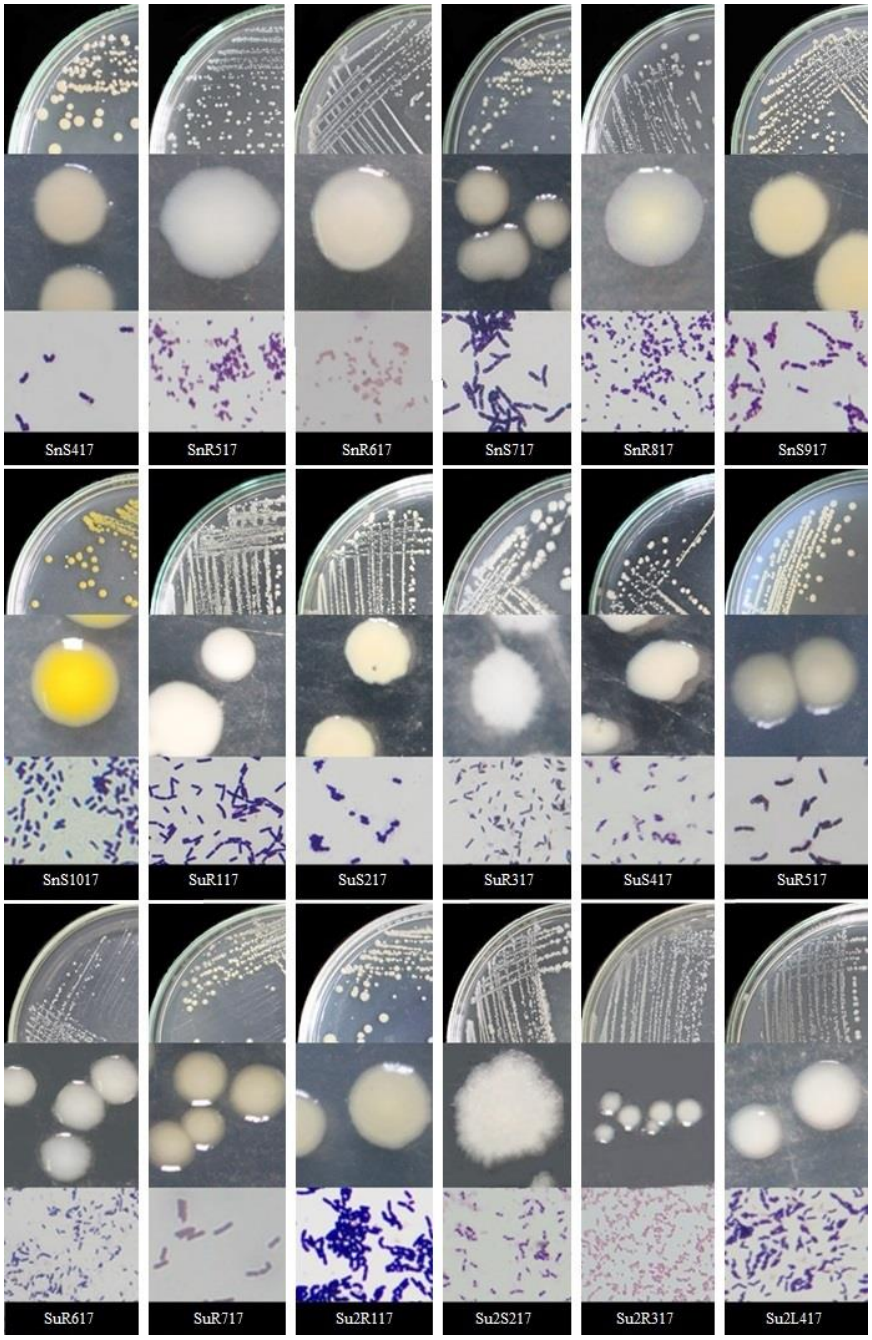

Figure 2(continue). Morphology of endophytic bacteria from healthy rice plant **(**Above = colony on NA, Middle = colony at 6.7X and Under = gram staining)

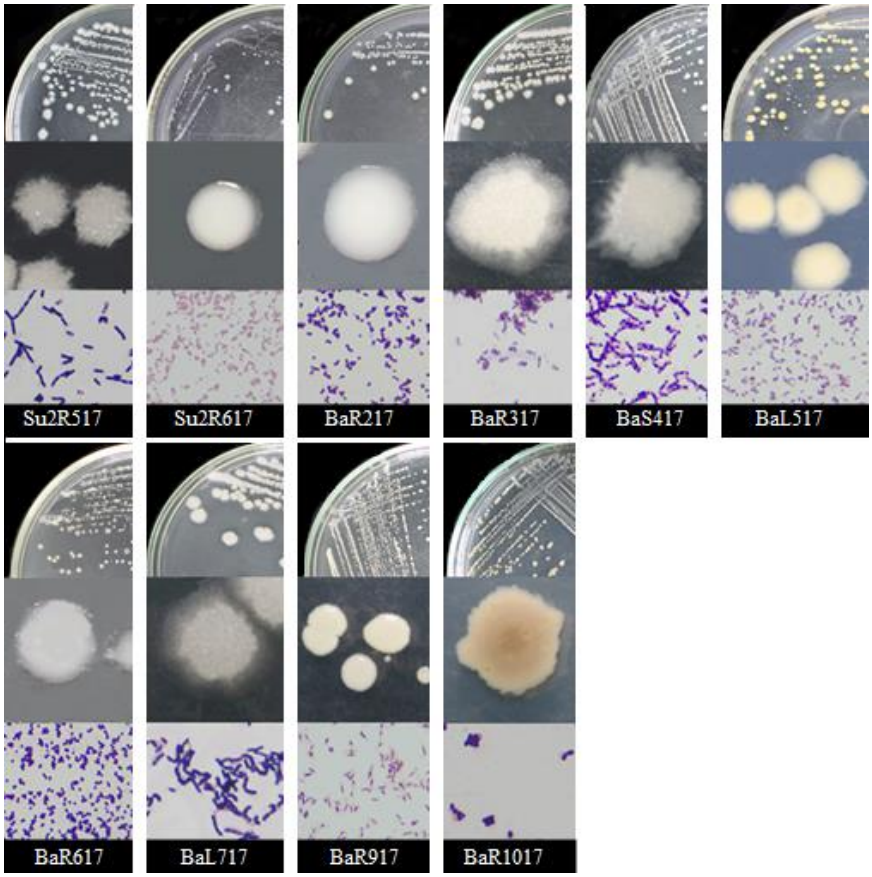

Figure 2 (continue). Morphology of endophytic bacteria from healthy rice plant **(**Above = colony on NA, Middle = colony at 6.7X and Under = gram staining)

Table 2. Effects of endophytic bacterie on the growth of rice seedlings.

Isolates	number of leave	Height	Survival of seedling	Shoot weight	Root weight	Total weight	%svi
1.control	2.2ab[1/]	13.05c	92.50ab	0.21abc	0.36cd	0.57bcd	100
2.BEdStUTI001	2.1ab	14.37abc	100a	0.19abc	0.36cd	0.56cd	107.74
3.BEdStUTI002	2.2a	13.25abc	97.50ab	0.20abc	0.38abcd	0.59abcd	111.61
4.BEdStUTI003	2b	13.00c	100a	0.18bc	0.36cd	0.54d	104.37
5.BEdStUTI004	2.15ab	13.12bc	97.50ab	0.17c	0.36cd	0.54d	101.76
6.BEdStUTI006	2.2a	13.07c	95.00ab	0.19abc	0.35cd	0.55cd	100.98
7.BEdStRBR001	2b	13.82abc	100a	0.19abc	0.38bcd	0.58abcd	111.59
8.BEdStRBR002	2b	13.90abc	100a	0.18bc	0.38abcd	0.57abcd	110.14

Isolates	number of leave	Height	Survival of seedling	Shoot weight	Root weight	Total weight	%svi
9.BEdStRBR003	2.15ab	13.10c	100a	0.18bc	0.36cd	0.55cd	105.82
10.BEdStRBR005	2b	14.68abc	92.50ab	0.23ab	0.37cd	0.60abcd	116.40
11.BEdStSPB001	2b	14.52abc	97.50ab	0.19abc	0.35d	0.54cd	105.33
12.BEdStSPB002	2.05ab	15.37a	97.50ab	0.19abc	0.35cd	0.54cd	105.33
13.BEdStSPB003	2.05ab	14.11abc	100a	0.23ab	0.40abcd	0.63abcd	121.21
14. BEdStSPB004	2.05ab	14.45abc	100a	0.21abc	0.38bcd	0.59abcd	114.47
15.BEdStSPB005	2.05ab	14.60abc	100a	0.18bc	0.39abcd	0.57abcd	111.11
16.BEdSTPB001_r	2.15ab	13.76abc	97.50ab	0.19abc	0.38bcd	0.57abc	107.86
17.SnR117	2.1ab	13.01c	92.50ab	0.18bc	0.35cd	0.54d	96.54
18.SnS217	2b	15.22abc	100a	0.19abc	0.35d	0.55cd	106.30
19.SnS317	2.05ab	13.15bc	100a	0.18bc	0.36cd	0.54d	104.37
20.SnS417	2b	14.09abc	95.00ab	0.18bc	0.38abcd	0.57bcd	104.18
21.SnR517	2.05ab	14.85abc	97.50ab	0.17c	0.35cd	0.54d	104.37
22.SnR617	2.05ab	14.80abc	100a	0.20abc	0.41abcd	0.62abcd	119.76
23.SnS717	2.05ab	13.15bc	100a	0.18bc	0.35d	0.53d	102.93
24.SnR817	2b	14.43abc	100a	0.18bc	0.35d	0.53d	102.45
25.SnS917	2.05ab	13.42abc	97.50ab	0.22abc	0.39abcd	0.61abcd	114.89
26.SnS1017	2b	13.02c	95.00ab	0.19abc	0.35d	0.53d	98.24
27.SuR117	2b	13.00c	97.50ab	0.18bc	0.35cd	0.54cd	102.23
28.SuS217	2.1ab	15.12abc	97.50ab	0.19abc	0.35d	0.55cd	97.07
29.SuR317	2.1ab	15.32ab	100a	0.21abc	0.42abcd	0.63abcd	121.21
30.SuS417	2.15ab	13.38abc	95.00ab	0.22abc	0.42abcd	0.64abc	117.89
31.SuR517	2.15ab	13.15bc	95.00ab	0.19abc	0.37cd	0.56cd	103.72
32.SuR617	2.05ab	14.18abc	97.50ab	0.22abc	0.45a	0.66ab	128.03
33.SuR717	2.15ab	13.00c	100a	0.18bc	0.35d	0.53d	102.93
34.Su2R117	2.15ab	13.03c	97.50ab	0.20abc	0.36cd	0.56cd	105.51
35.Su2S217	2.1ab	13.90abc	92.50ab	0.23ab	0.38abcd	0.61abcd	109.89
36.Su2R317	2.1ab	14.33abc	90.00b	0.21abc	0.38abcd	0.60abcd	103.89
37.Su2L417	2b	13.78abc	100a	0.24a	0.37cd	0.61abcd	117.84
38.Su2R517	2b	13.62abc	100a	0.20abc	0.36cd	0.57bcd	109.66
39.Su2R617	2.05ab	14.07abc	100a	0.20abc	0.37cd	0.57bcd	109.66
40.BaR217	2.05ab	14.75abc	97.50ab	0.21abc	0.45ab	0.66ab	128.42
41.BaR317	2.1ab	13.24abc	100a	0.19abc	0.35cd	0.55cd	105.82
42.BaS417	2.15ab	13.64abc	97.50ab	0.19abc	0.35cd	0.55cd	103.17
43.BaL517	2b	13.82abc	100a	0.19abc	0.40abcd	0.60abcd	115.92
44.BaR617	2b	14.47abc	95.00ab	0.21abc	0.36cd	0.58abcd	106.01
45.BaL717	2.1ab	14.70abc	100a	0.20abc	0.40abcd	0.62abcd	119.28
46.BaR917	2.1ab	13.73abc	100a	0.24a	0.43abc	0.67a	128.90
47.BaR1017	2.1ab	13.24abc	97.50ab	0.19abc	0.43abc	0.62abcd	116.30

[1/]Means in the same column with different letters are singnificant different at P =0.05 , according to Duncan's Multiple range test (DMRT)

Inhibitory activity against rice blast pathogen

All isolates of endophytic bacteria in this experiment was also tested for their efficiency to inhibit *P.oryzae* by dual culture technique. There were 5 isolates could inhibit more than 50%, those were SuS217, BarR917, BaS417, SuR317 and Su2S217, which referred to antagonistic isolates. Their percentage of inhibition was 58.74, 61.11, 64.86, 66.66 and 66.80, respectively. The rest of isolates had percentage of inhibition between 9.72-49.02%. (Figure 3 and Table 3)

Table 3. Effects of endophytic bacterie on growth of rice seedlings.

Isolates	Inhibitory against *P.oryzae*	
	Diameter of colony (cm.)	Growth inhibition (%)
1.BEdStUTI001	7.53	16.25
2.BEdStUTI002	7.52	16.38
3.BEdStUTI003	6.88	23.47
4.BEdStUTI004	7.15	20.55
5.BEdStUTI006	7.25	19.44
6.BEdStRBR001	5.71	36.52
7.BEdStRBR002	7.75	13.88
8.BEdStRBR003	5.71	36.52
9.BEdStRBR005	7.41	17.63
10.BEdStSPB001	8.12	9.72
11.BEdStSPB002	5.40	40.00
12.BEdStSPB003	5.18	42.36
13. BEdStSPB004	6.5	27.77
14.BEdStSPB005	4.58	49.02
15.BEdSTPB001_r	6.85	23.88
16.SnR117	5.50	38.88
17.SnS217	5.50	38.88
18.SnS317	6.18	31.25
19.SnS417	7.15	20.48
20.SnR517	5.87	34.72
21.SnR617	6.42	28.61
22.SnS717	5.35	40.55
23.SnR817	6.28	30.13
24.SnS917	6.21	30.97
25.SnS1017	7.00	22.22
26.SuR117	5.77	35.83
27.SuS217	3.73	58.47
28.SuR317	3.00	66.66
29.SuS417	5.71	36.52
30.SuR517	6.08	32.36
31.SuR617	7.78	13.47
32.SuR717	5.91	34.30
33.Su2R117	7.61	15.41

Isolates	Inhibitory against *P.oryzae*	
	Diameter of colony (cm.)	Growth inhibition (%)
34.Su2S217	2.98	66.80
35.Su2R317	6.55	27.22
36.Su2L417	5.77	35.83
37.Su2R517	5.90	34.44
38.Su2R617	6.96	22.63
39.BaR217	6.30	30.00
40.BaR317	6.61	26.52
41.BaS417	3.16	64.86
42.BaL517	6.82	24.16
43.BaR617	8.03	10.69
44.BaL717	5.23	41.80
45.BaR917	3.50	61.11
46.BaR1017	6.60	26.66

[1/]Diameter of colony*P.oryzae* at 14 day

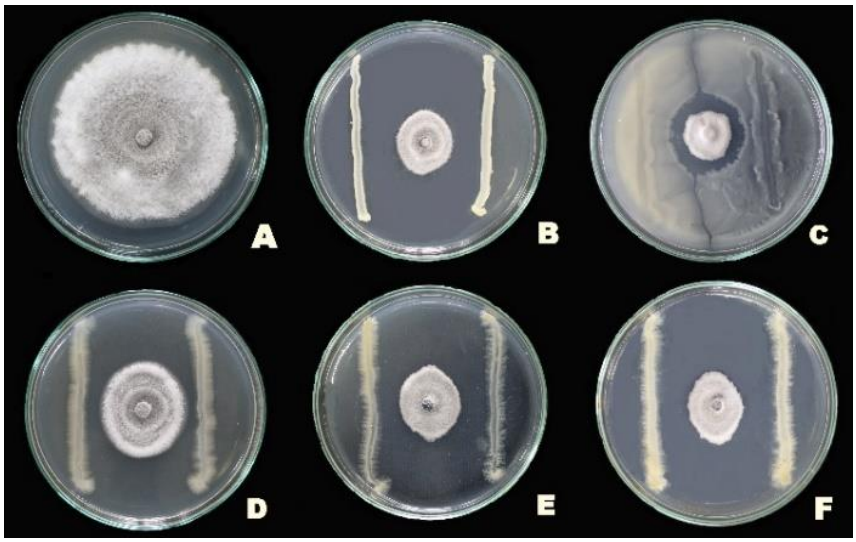

Figure 3 Dual-culture of five isolates inhibit more than 50 percentage (A=control, B = BarR917, C=BaS417, D= SuR317, E= SuS217 and F= Su2S217

Therefore, the screening of endophytic bacteria that promote plant geowth and inhibit rice blast pathogen was considered among beneficial isolates and antagonistic isolates.The most interesting isolates were BaR917 because of the highest %SVI and the highest percentage of inhibition of rice blast disease. Therefor, BaR917 has been selected as a potential isolate for further study on seed bio-priming.

Discussion

Isolation and screening of endophytic bacteria to select beneficial bacteria for growth promotion and control plant pathogens is an interesting topic, because there are a lot of research in several areas. (Hardoim *et. al*., 2008; Yang *et. al*., 2008; Muthukumar and Venkatesh, 2013; Koohakan and Konrangdee, 2015). In this study, we obtained 31 recent isolate from healthy rice, those were SnR117, SnS177, SnS177, Sn417, SnR1717, SnR177, SnS717, SnR817, SnS917, SnS1017, SuR117, SuS217, SuR317, SuS417, SuR517, SuS617, SuR717, Su2R117. Su2S217, Su2R317, Su2L417, SuS2R517, Su2R617, BaR217, BaR517, BaL517, BaS417, BaR617, BaL717, BaR917, and BaR1017. Base on ordinary study, most isolates were gram-positive (27 isolates) and the other was gram-negative (4 isolates) and showed different in morphological characteristics.

Selection of endophytic bacteria for plant growth promotion is important, because associated bacteria could be interact to plant either positive or negative. Therefore, we have to select only the positive isolate that benefit to plant in the category of growth promotion to plant and suppression to the pathogen. This experiment used %SVI to screen the growth promotion isolates and found that it had 23 recent isolates that was highest % SVI, which higher than control between 2.23-28.90% they were includings; SnS217, SnS317, Sn417, SnR517, SnR617, SnS717, SnR817, SnS917, SuR117, SuR317, SuS417, SuR517, SuS617, SuR717, Su2R117, Su2S217, Su2R317, Su2L417, SuS2R517, Su2R617, BaR217, BaR517, BaL517, BaS417, BaR617, BaL717, BaR917 and BaR1017. According to Ji *et al.* (2014) reported 576 isolates of endophytic bacteria from 10 rice cultivars were isolated.The athor found that it has 12 isolates that can promote the growth of rice and inhibited *Fusarium oxysporum* and *Rhizoctonia solani*, a major rice pathogen. It can be seen that endophytic bacteria are not only useful in promoting plant growth. Some isolates may also have the ability to control plant pathogens.

Therefore, this study was conducted to study the bio-activity of 46 endophytic bacteria examined for growth promotion on rice that mentioned above and antagonist against *P. oryzae*. This results found that all isolates could be inhibit the growth of pathogen. The best 5 isolates were SuS217, BarR917, BaS417, SuR317 and Su2S217, which the percentage of inhibition was 58.74, 61.11, 64.86, 66.66 and 66.80%, respectively. Several studies have reported that the use of endophytic bacteria can inhibit pathogens. (Nejad and Johnson, 2002; Pageni *et al.*, 2014; Koohakan and Konrangdee, 2015) In conclusion, the results of this study show that endophytic bacteria are interesting for use in biological control and promote plant growth and could be possible for study on seed bio-priming to develop rice production in the future.

Acknowledgement

This research was supported by Geduate Research Fund from Faculty of Agricultural Technology, King Mongut's Institute of Technology Ladkrabang, Bangkok, Thailand.

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(Received: 24 October 2017; accepted: 25 November 2017)